

Monitoring the accumulation and dissipation of the  
photosensitizer protoporphyrin IX during standard dermatological  
methyl-aminolevulinate photodynamic therapy utilizing  
non-invasive fluorescence imaging and quantification

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## **ABSTRACT**

**Background:** Dermatological methyl-aminolevulinate photodynamic therapy (MAL-PDT) is utilized to successfully treat dermatological conditions. This study monitored fluorescence changes attributed to the accumulation and destruction of the photosensitizer, protoporphyrin IX (PpIX), at several different stages during the first and second treatments of clinical dermatological MAL-PDT.

**Methods:** A commercially available, non-invasive, fluorescence imaging system (Dyaderm, Biocam, Germany) was utilized to monitor fluorescence changes during the first and second MAL-PDT treatments in seventy-five lesions.

**Results:** The clinical data indicated statistically significant increases in fluorescence within lesions following the application of MAL for both treatments ( $P<0.001$  and  $P<0.01$  respectively) and subsequent statistically significant decreases in fluorescence within the lesions following light irradiation for both treatments ( $P<0.001$  and  $P<0.01$  respectively) whilst normal skin fluorescence remained unaltered.

Lesions receiving a second treatment accumulated and dissipated significantly less PpIX ( $P<0.05$ ) than during the first treatment. No significant differences were noted in PpIX accumulation or dissipation during MAL-PDT when gender, age, lesion type and lesion surface area were considered.

**Conclusions:** It can therefore be concluded that PpIX fluorescence imaging can be used in real-time to assess PpIX levels during dermatological PDT. Similar observations were recorded from the three currently licensed indications indicating that the standard 'one size fits all' protocol currently employed appears to allow

adequate PpIX accumulation, which is subsequently fully utilized during light irradiation regardless of patient age, gender or lesion surface area.

## **INTRODUCTION**

Photodynamic therapy (PDT) enables the selective destruction of tumor tissue preserving the majority of the surrounding normal tissue (1, 2) and so has huge potential as a cancer therapy. PDT induces the production of highly localized reactive oxygen species (ROS) which contribute to cellular damage and ultimately cell death via apoptotic, necrotic and autophagic mechanisms (3). The production of ROS during PDT is completely reliant on the presence of three critical components; a photosensitizer, light of the appropriate wavelength and molecular oxygen (3). The absence or mistiming of just one of these components results in no ROS production /cell death (4).

PDT is now widely employed in treating a variety of different carcinomas, with numerous different photosensitizers being available (2, 5, 6). PDT has found a particular niche in the treatment of non-melanoma skin cancers (NMSC) and associated pre-cancers which are the most prevalent malignancy in the Caucasian population. In the United States more than one third of all adult cancers are NMSC, with between 900,000 to 1,200,000 new cases occurring per year (7). PDT for NMSC utilizes a topical pro-drug (either aminolevulinic acid (ALA) or an ester derivative) which results in the accumulation of the endogenous photosensitizer, protoporphyrin IX (PpIX), within the tumor cells. The topical pro-drug is converted enzymatically via

the heme biosynthesis pathway (8) to PpIX (the immediate precursor to heme), which accumulates preferentially due to differences in the stratum corneum overlying tumors improving prodrug penetration into the lesion and alterations in various enzyme activities (8). The accumulated PpIX is then activated utilizing a red light (635 nm) and providing sufficient tissue oxygen is present, ROS are produced within the diseased cells resulting in cellular damage and subsequent cell death (8).

Topical PDT with ALA (or an ester derivative, in the UK the methyl derivative methylaminolevulinate (MAL) is the licensed pro-drug (Metvix®, Galderma, UK)) has proved to be successful in the treatment of NMSC (including superficial basal cell carcinoma (sBCC) and Bowen's disease (BD)) and associated pre-cancers (e.g. actinic keratosis (AK)). The clinical clearance rates are similar to standard treatment methodologies for these conditions (e.g. surgical excision and curettage) (9-16). Furthermore PDT negates several other issues that commonly arise in the standard treatment of these indications, including lesion size and multiplicity as well as also resulting in a better cosmetic outcome (17).

The accumulation and dissipation of the endogenous photosensitizer, PpIX, can be followed by exploiting the characteristic fluorescence properties of PpIX (18).

Excitation of PpIX with blue light (410 nm) results in detectable fluorescence in the red region (635 nm and 700 nm) (19). The fluorescent properties of PpIX are commonly employed in photodiagnosis, aiding clinicians in diagnosis and demarcation for excision (20, 21). In pre-clinical studies the fluorescent properties of PpIX are utilized

to follow changes in the photosensitizer concentration (22-25). Whilst non-invasive techniques are preferred, clinically to date the majority of information about accumulation of the photosensitizer derives from chemical extraction conducted on excised tissue followed by high performance liquid chromatography (26, 27). This invasive technique does not enable the photosensitizer to be followed during real-time, it simply provides information about PpIX concentration at one specific point following the process being terminated. The non-invasive commercially available imaging system employed within this study (Dyaderm, Biocam, Germany) is utilized by several groups for photodiagnosis of skin lesions (28, 29). Following extensive validation, this system was considered to be capable of monitoring changes in PpIX during real-time MAL-PDT (30). Whilst numerous factors hinder reproducibility in fluorescence imaging (31) it was shown that consistent application resulted in reproducible images been acquired of normal human skin (30).

This study utilized the Dyaderm to follow MAL-PDT treatment of licensed indications (AK, BD and sBCC) in the dermatology PDT clinic at Royal Cornwall Hospital (Truro, UK). A positive correlation has been previously demonstrated between PpIX photobleaching and cellular damage (22, 25, 32, 33), indicating that the greater the level of photobleaching the more efficacious the PDT treatment. This finding indicated that greater PpIX accumulation increases the potential of the therapy as more PpIX is available for activation during irradiation. Therefore one of the main modalities of treatment enhancement focuses around increasing the level of PpIX within cells utilizing a variety of different techniques including lesion preparation (34),

alternative prodrugs (35), penetration enhancers (36) and iron chelators (24, 37).

Hence the identification of general factors that may limit or enhance accumulation and dissipation of PpIX is important to establish in order to enable further understanding of the treatment process, and further investigation may result in more individualized treatment programs being developed in the future.

## **MATERIALS AND METHODS**

*Fluorescence imaging:* The study employed a commercially available non-invasive fluorescence imaging system (Dyaderm, Biocam, Germany). The system consisted of a Xenon flash light source that has a custom bandpass filter (370-440 nm) that alternated between white and blue light (370-440 nm) and a 12-bit Sony charge coupled device (CCD) camera combined in one adjustable arm coupled to a Pentium IV computer equipped with custom-made image capturing software (Dyaderm Pro v2, Biocam, Germany). Seven light pulses per second were released from the camera to the area of interest, and the returning light was collected by the CCD camera (exposure time 100  $\mu$ s) which utilized a special Schott GG 455 longpass filter to exclude the excitation light (370-440 nm). The red pixels of the CCD camera (spectral sensitivity of which at 630 nm is between 85% and 90%) were used to generate a fluorescence image from the red spectrum fluorescence emitted from the excited PpIX. In this way, a normal colored image (from the white light) and a fluorescence image (from the blue light 370-440 nm) were simultaneously collected and processed by the system in real time. The images acquired were magnified by a factor of three.

PpIX is not the sole fluorophore within the skin to be activated by blue light; lipopigments and flavins both emit light in the green spectrum when excited with blue light. The autofluorescence was also recorded by the camera, enabling changes in autofluorescence to be considered during real time MAL-PDT, these were subtracted from the PpIX image produced to ensure the sole changes recorded resulted from changes in the concentration of the porphyrins. To ensure the stability of the camera a synthetic PpIX fluorescence standard (Biocam, Germany) was imaged on each clinic day to ensure the continuity of the system.

*Clinical data capture:* All patients attending for their first routine MAL-PDT appointment for licensed indications (AK and biopsy proven BD and sBCC) at our Dermatology Department (Royal Cornwall Hospital, Truro, UK) were informed about this ethically approved study (Cornwall and Plymouth Research Ethics Committee) and subsequently recruited. The seventy-five recruited patients were treated as for a normal MAL-PDT clinic, with any crust overlying the lesion prior to treatment gently removed with curettage. The topical pro-drug MAL (commercially available as Metvix®, 160 mg/g MAL, Galderma, UK) was then applied to the lesion at approximately 1 mm thickness, with a 5 mm border. The lesion was then covered with an occlusive dressing for the three hour application period. Any excess MAL was then wiped away and the lesion irradiated with a standard light protocol (Aktelite, Photocure, Norway, 635 +/- 5 nm, 37 Jcm<sup>-2</sup>, 80 mWcm<sup>-2</sup>, positioned 5-8 cm above the lesion). Lesions were covered with a dressing to prevent any subsequent light exposure as the area was photosensitive for approximately 24 hours. The patients' lesion was imaged at three distinct time points during the treatment

process; prior to the application of MAL, after the three hour application of MAL and immediately following light irradiation. Only one lesion was imaged per patient to increase statistical power. The imaging followed the standard protocol derived previously within our group which enabled reproducible images to be acquired (30). Details including the patients age, gender, lesion type, location and size were all recorded prior to commencement of imaging. The lesions were treated as per the National Institute of Clinical Excellence (NICE) guidelines (38) although all lesion types received two identical treatments nine days apart here. Twenty five of each licensed lesion type were recruited and monitored for the two PDT treatments. When the treatment was complete the images were exported for further image analysis.

*Data analysis:* The images were exported as Bitmaps (640\*512 pixels - to avoid data loss) and analyzed in NIH ImageJ software (<http://rsb.info.nih.gov/ij/>). The images were analyzed from the same point within the lesion, as previous studies indicated that this lowered the coefficient of variance (data not shown) and the mean grayscale values recorded for each time point, enabling quantitative analysis of PpIX levels. These data were analyzed for statistical significance via either the repeated measures ANOVA or the paired student's t-test, comparing and contrasting the fluorescence changes observed during the first and second PDT treatment cycles, the distribution of the photosensitizer and any relationships between changes in accumulation/dissipation and patient gender (42 males and 33 females), patient age (patient age range 46-98 years, mean age 77 years) and lesion size. Fluorescence distribution was also monitored in all lesions by measuring the grayscale values at fourteen locations spiraling out from the lesion centre.



## RESULTS

### Fluorescence changes within licensed indications during the first and second MAL-PDT treatments

Prior to image acquisition the synthetic PpIX standard was monitored and over the course of this study no significant difference in the grayscale intensity was recorded ( $P=0.950$ , data not shown). The images acquired from the licensed indications indicated that the majority underwent detectable fluorescence changes during the first and second MAL-PDT treatments (Figure 1). Subsequent image analysis demonstrated a significant increase in fluorescence intensity within all three distinct lesion types following the three hour MAL application ( $P<0.001$  and  $P<0.01$  respectively, Figures 2a and b). Following light irradiation a significant decrease ( $P<0.001$  and  $P<0.01$  respectively, Figures 2a and b) in the grayscale intensities was observed within all three lesion types following light irradiation during both the first and second treatments. In contrast the area of normal skin monitored concurrently showed no significant changes in fluorescence during the treatment procedure ( $P=0.839$  (1<sup>st</sup> treatment) and  $P=0.549$  (2<sup>nd</sup> treatment), data not shown). For both the first and second treatments no significant differences were observed between the distinct lesion types in terms of the accumulation (ANOVA; 1<sup>st</sup> treatment  $P=0.950$ ; 2<sup>nd</sup> treatment  $P=0.710$ ) and dissipation (ANOVA; 1<sup>st</sup> treatment  $P=0.740$ ; 2<sup>nd</sup> treatment  $P=0.270$ ) of the photosensitizer (Figure 3). A positive correlation existed between the total PpIX fluorescence after the three hours of MAL application and the fluorescence decrease during light irradiation ( $r^2=0.53$ ; data not shown). The level of PpIX accumulation during both the first and second treatments after three hours MAL

application was approximately equal ( $P>0.250$ ) to the dissipation observed during light irradiation on each occasion (Figure 4). However during the second treatment the total accumulation/dissipation of PpIX observed was statistically significantly lower than when compared to the initial treatment ( $P<0.01$  and  $P<0.05$  respectively for all three indications separately (Figure 3)). Furthermore the area of fluorescence within the lesion was found to be significantly smaller ( $P<0.01$ ) on the second PDT treatment (Figure 5) as could be observed visually from the images (Figure 1). The distribution of the photosensitizer was monitored within these lesions by analyzing the grayscale intensity of the lesion as you moved away from the centre. PpIX distribution varied greatly, with an approximately equal split between lesions demonstrating no variation ( $n=32$ , 43%) and lesions demonstrating significant ( $P<0.01$ ) variation ( $n=43$ , 57%). Lesions with a surface area of less than  $50 \text{ mm}^2$ , were significantly ( $P<0.01$ ) more likely to have homogeneously distributed PpIX (100% versus 21%).

### **Relationship of PpIX accumulation and dissipation with patient age, gender and lesion size**

The patients were split into four distinct age brackets on the basis of their age (under 60 ( $n=14$ ), 60-70 ( $n=16$ ), 70-80 ( $n=21$ ) and over 80 ( $n=24$ ) years of age) and the accumulation and dissipation of PpIX fluorescence were compared. The different age groups showed no significant differences in terms of PpIX accumulation ( $P=0.270$ ) and dissipation ( $P=0.310$ ) (Figure 6). A direct comparison was also made in terms of accumulation between the youngest and oldest groups but no significant difference was observed (unpaired t-test,  $P=0.100$ ). As observed previously PpIX accumulation

approximated to PpIX dissipation in three of the four age groups ( $P=0.131$ ,  $P=0.413$  and  $P=0.09$  for the under 60s, 60-70 and 70-80 age groups respectively); in contrast the accumulation in the over 80 age group was observed to be significantly higher than the dissipation ( $P < 0.05$ ). Patient gender ( $n=42$  (56%) males and  $n=33$  (44%) females) was not observed to significantly alter accumulation ( $P=0.791$  and  $P=0.924$ ) or dissipation of PpIX ( $P=0.391$  and  $P=0.868$ ) during both the first and second PDT treatments respectively (Figure 7). No correlations were observed between the surface area of the lesion and the MAL-PDT induced changes in fluorescence within these lesions ( $r^2 = 0.02$  for accumulation and  $r^2 = 0.01$  for dissipation; Figures 8a & 8b respectively).

## DISCUSSION

This investigation has demonstrated the capacity of a non-invasive imaging system (Dyaderm) to detect fluorescence changes during standard clinical dermatological MAL-PDT for licensed indications (AK, BD and sBCC). The fluorescence changes arise as a result of the accumulation and dissipation of porphyrins and previous studies have indicated that photosensitizer PpIX is the dominant porphyrin to accumulate within cells after MAL application (26, 27); additionally we have tested the imaging system ourselves utilizing a synthetic PpIX standard which indicated that the mean grayscale values calculated related to the PpIX concentration (30).

The three licensed lesion types were each observed to undergo two distinct changes in fluorescence during the first and second PDT treatments in the majority of cases, whilst areas of surrounding normal skin remained unaltered. Firstly following the

three hour MAL application a statistically significant increase in fluorescence was observed during both the first and second PDT treatments (Figures 1 & 2). This increase in fluorescence was attributed to the conversion of MAL via the heme biosynthesis pathway to the photosensitizer PpIX (9). The second change in fluorescence was observed immediately following light irradiation where statistically significant decreases in fluorescence were detected during both the first and second treatments (Figures 1 & 2). The decrease in fluorescence during light treatment was attributed to the photobleaching of PpIX as a result of the production of singlet oxygen (8). The fluorescence changes monitored correlated well with our current understanding of the accumulation and dissipation of PpIX during standard dermatological MAL-PDT (8, 39). The level of accumulation and dissipation of fluorescence in individual lesion types during MAL-PDT was approximately equal with no significant difference observed between these two measurements (Figure 3). This indicated that the light irradiation phase was effectively photobleaching all the accumulated PpIX within the cells of the lesions. This suggested that providing three hours was the optimal application time for MAL (40), then maximal treatment potential was reached in the majority of lesions and increasing the light dose would be unnecessary as all the PpIX accumulated was already being fully utilized, as no excess PpIX remained after the irradiation period. Furthermore all three licensed lesion types were observed to respond in a similar manner (Figure 4). However a larger sample size (100 of each type of lesion) would be required to exclude a type II error. The accumulation and dissipation of PpIX in the different histological lesions might be expected to be similar as the conditions are derived from cells within the

epidermis, treated in an identical manner with a standard licensed and well evidenced protocol and response rates to this PDT treatment method are similar (7, 13, 41-43).

It is important to note that inter patient variation was large and whilst the data presented here represents all of the lesions monitored, a small number of lesions did not follow the general trend of significant accumulation and dissipation of fluorescence. Within the data set there were several examples of lesions which either showed limited accumulation and subsequent dissipation of PpIX or demonstrated significant accumulation but limited dissipation of the photosensitizer. These anomalies could simply be attributed to inter patient variation; however an underlying cause seemed a more likely explanation. Limited PpIX accumulation could be due to inadequate lesion preparation, the application of insufficient pro-drug to the area and/or incorrect diagnosis (although sBCC and BD lesions were biopsy proven). Our data collection has also analyzed the role of the practitioner as our service is run with several different practitioners (specialized PDT nurses). However, no significant differences were observed between these different practitioners in terms of accumulation and dissipation of PpIX (data not shown) suggesting whilst minor variations in treatment procedure existed they did not significantly alter the potential efficacy of the treatment. Several factors may be involved in the lesions which underwent significant accumulation but limited photobleaching during light irradiation, the light placement and the relationship between PpIX concentrations and photobleaching. The light was placed between 5-8 cm away from the lesion surface depending on the nurse practitioner and this variation could potentially result in

approximately a 10% alteration in the light dosage delivered. However, the dissipation of PpIX was noted to be similar for all practitioners. Previous *in vitro* studies have indicated that higher PpIX concentrations undergo lower levels of photobleaching (22, 44) potentially as a result of saturation of the primary photosensitizer binding sites at high concentrations of PpIX therefore PpIX localizes at secondary sites which either protect PpIX from singlet oxygen or make it less photoactive. However within this data set a positive correlation was observed between the total PpIX fluorescence after three hours of MAL application and PpIX photobleaching. Alternatively other factors may also be involved, especially the general health of the patient.

The second MAL-PDT treatment resulted in lesions undergoing significant changes in fluorescence (Figure 2b) indicating that the second treatment was worthwhile. However, the accumulation and dissipation of the photosensitizer was significantly lower than observed for the first treatment (Figure 3). This indicated that the first treatment effectively destroyed the majority of cancerous and pre-cancerous tissue, whilst the second treatment removed the remainder of the diseased cells. This was further represented by the decrease observed in the size of the fluorescent area following the application of MAL for the second treatment cycle (Figure 5). The accumulation of PpIX is known to be preferential in tumor tissue when compared with normal skin due to the proposed differences in stratum corneum thickness and altered enzyme activities (8) hence this data suggests that during the second PDT treatment cycle there were areas of normal skin within the lesion borders (to which the prodrug

was applied) which no longer preferentially accumulated PpIX in the three hour application time. In fact in our experience there were several cases where visually the lesion had improved so significantly following just the first PDT treatment cycle that it was barely visible on the second treatment.

The distribution of the photosensitizer following the three hour application of MAL was also studied for lesions undergoing the first PDT treatment cycle. The photosensitizer distribution was observed to not alter significantly across the lesion area after the three hours of MAL application for 43% of the lesions studied suggesting a homogenous photosensitizer distribution as previously noted in basal cell carcinomas (36, 40). The remaining 57% of lesions demonstrated heterogenous PpIX fluorescence distribution correlating with previously published observations (45-48). On further analysis of the data the surface area of the lesion appeared to contribute to the distribution of the photosensitiser with smaller lesions tending to demonstrate a more homogenous fluorescence distribution. The distribution of the photosensitizer is of critical importance as it determines the sites of ROS production providing light and oxygen are readily available. It might therefore be interesting to determine in future investigations whether lesions with homogenous fluorescence during the first PDT treatment respond better to PDT due to a more consistent response across the entire lesion. The photosensitiser distribution was not studied for the second treatment cycle as we had previously shown that much less photosensitiser accumulated during this second cycle and that the PpIX localized to specific areas which had not been successfully cleared during the first treatment cycle.

Patient gender was examined as it was proposed that differences in stratum corneum thickness and microvasculature may alter the accumulation and dissipation respectively. Previous studies have indicated a weak negative correlation between the thickness of the stratum corneum and the PpIX fluorescence observed after topical prodrug application (46). These data demonstrating a difference between stratum corneum thickness in males and females is controversial, with data mainly supporting no significant differences in the stratum corneum thickness (49-51). Our data demonstrated no significant differences in PpIX accumulation and dissipation for males and females (Figure 7) indicating that gender does not significantly alter the susceptibility of the skin for MAL uptake or subsequent photosensitizer destruction. However, power calculations indicated 600 males and 600 females would be required to rule out statistical error in this respect. The study indicated that the patients' age did not significantly alter the accumulation and dissipation of PpIX during the first treatment cycle of MAL-PDT (Figure 6). However there was a small difference between the two extremes of age which was approaching but did not achieve statistical significance with this sample size. This may be due to the thinning of the stratum corneum over time and therefore more elderly patients may be more efficient at taking up the pro-drug, MAL, enabling higher concentrations of PpIX to accumulate within the tumor cells. This would be interesting to investigate further, however one limitation of this data is that relatively few patients fall in the under 60 age bracket (19% here) with even fewer under 50 (6% here), hence indicating that investigating extremes of age in terms of accumulation would probably lack statistical power. Perhaps predictably in light of the data already presented here (with PpIX dissipation



mimicking PpIX accumulation) no significant difference was observed when studying PpIX dissipation during light irradiation in licensed indications in different age brackets (Figure 6). With the exception of the over 80s age bracket, where accumulation was significantly greater than dissipation of PpIX, accumulation approximated to dissipation within the age groups (Figure 6). Significant further study would be required to rule out a type II error. However, it is feasible that more elderly patients struggled to completely dissipate the PpIX during light irradiation as a result of the altered cutaneous microvasculature that occurs with age, with a general trend towards decreased cutaneous perfusion in older individuals (52-55), which potentially lowers oxygen availability, therefore potentially reducing oxygen-dependent photobleaching. No correlation was observed between the surface area of the lesion and the relative levels of accumulation and dissipation of PpIX during MAL-PDT (Figures 8a & 8b). This was expected as the entire surface of the lesion was covered with MAL and the whole lesion irradiated in accordance with the standard treatment protocol.

Previous studies have shown the relationship between photobleaching of PpIX and cellular damage (22, 25, 33, 39) and previous work within our group has linked changes in fluorescence intensity recorded with the non-invasive imaging system to clinical outcome (56). The greater the photobleaching of PpIX the greater the likelihood a complete clinical clearance of the lesion will occur. These data presented suggest that all licensed indications responded in a similar manner in terms of PpIX accumulation and dissipation during MAL-PDT regardless of patient age, gender or lesion surface area.

In this study, we have observed that statistically significant changes in PpIX fluorescence occur during both the first and second MAL-PDT treatments and that all three licensed indications (AK, BD and sBCC) responded in a similar manner, supporting the one treatment regimen fits all protocol. We have also demonstrated that whilst the second treatment undergoes statistically smaller changes in fluorescence intensity than during the first treatment, importantly however, these changes were noted to be significant indicating that application of a second treatment was efficacious/worthwhile. Furthermore it was noted that with the sample size investigated, patient age, gender and lesion size did not appear to significantly alter accumulation and dissipation of the photosensitizer. These initial data provide an insight into photosensitizer accumulation and dissipation during dermatological MAL-PDT and with further application may be used in the future to evaluate various enhancement modalities.

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## FIGURE CAPTIONS

**Figure 1.** Fluorescence images acquired from a superficial basal cell carcinoma lesion located on the chest of a female patient undergoing the two MAL-PDT treatments. The images were taken at three distinct time points during MAL-PDT (from left to right: prior to the application of MAL, after the three hour MAL application and immediately after light irradiation). The PpIX fluorescence intensity is represented by the color bar at the bottom of the figure which increases from left to right.

**Figure 2.** Bar chart indicating the changes in PpIX fluorescence intensity for licensed lesions undergoing a) the first MAL-PDT treatment and b) the second treatment of MAL-PDT between all lesions at each time point. The error bars represent the standard deviations of the data.

**Figure 3.** Bar chart illustrating the average accumulation and dissipation of PpIX fluorescence for AK, BD and sBCC lesions during the first and second MAL-PDT treatments. \* and + represents a statistically significant decrease in the average accumulation and dissipation observed during the second treatment cycle at  $P < 0.01$  and  $P < 0.05$  respectively. The error bars represent the standard deviations of the data.



**Figure 4.** Bar chart comparing the accumulation and dissipation of PpIX fluorescence during the first and second MAL-PDT treatments of licensed lesions. The error bars represent the standard deviations of the data.

**Figure 5.** Bar chart illustrating the mean pixel area of PpIX fluorescence recorded from images acquired after the 3 hour MAL application on both the first and second MAL-PDT treatments (n = 75). \* indicates statistical significance ( $P < 0.01$ ). The error bars represent the standard deviations of the data.

**Figure 6.** Bar chart illustrating the mean accumulation and dissipation of the photosensitizer PpIX during real-time MAL-PDT for different patient age groups undergoing the first treatment. + indicates a significant difference between accumulation and dissipation of the photosensitizer in the over 80s group ( $P < 0.05$ ). The error bars represent the standard deviation of the data.

**Figure 7.** Bar chart illustrating the mean PpIX accumulation and dissipation by gender during dermatological MAL-PDT for licensed indications (n=75). The error bars represent the standard deviations of the data.

**Figure 8.** Linear regression graphs illustrating the relationship between the surface area of the lesion and a) PpIX accumulation and b) PpIX dissipation during MAL-PDT. The line represents the regression with coefficients of a) 0.02 and b) 0.01 respectively.

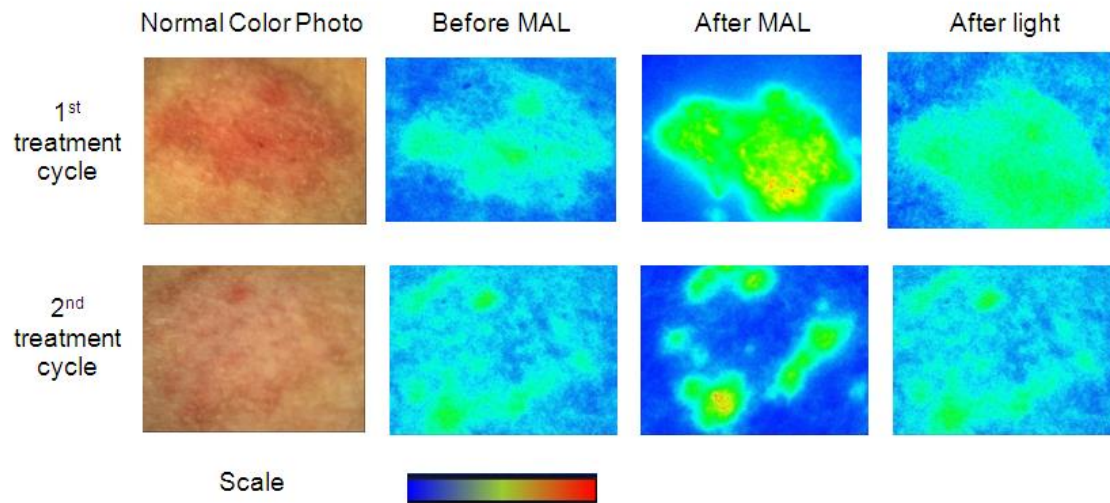
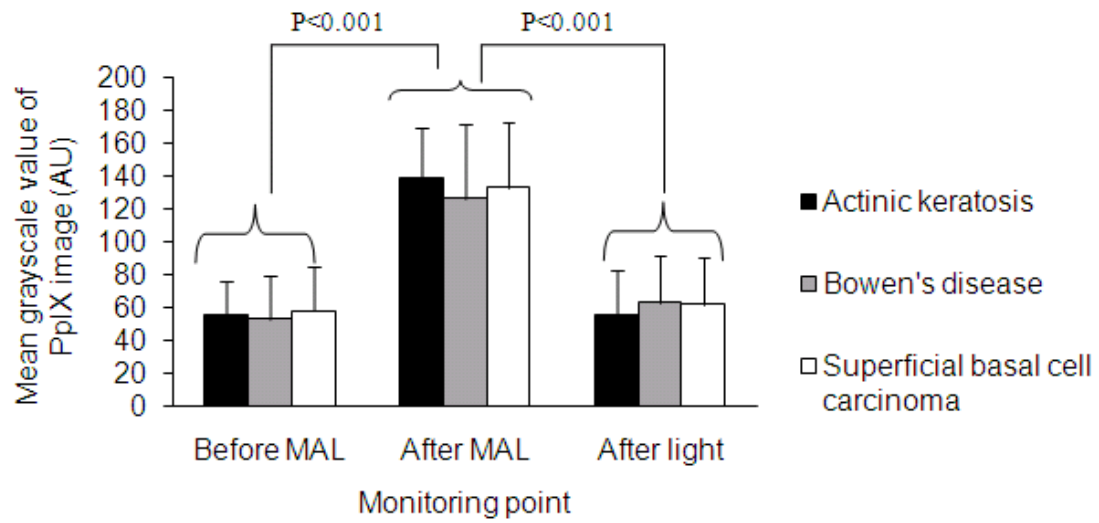


Figure 1

a



b

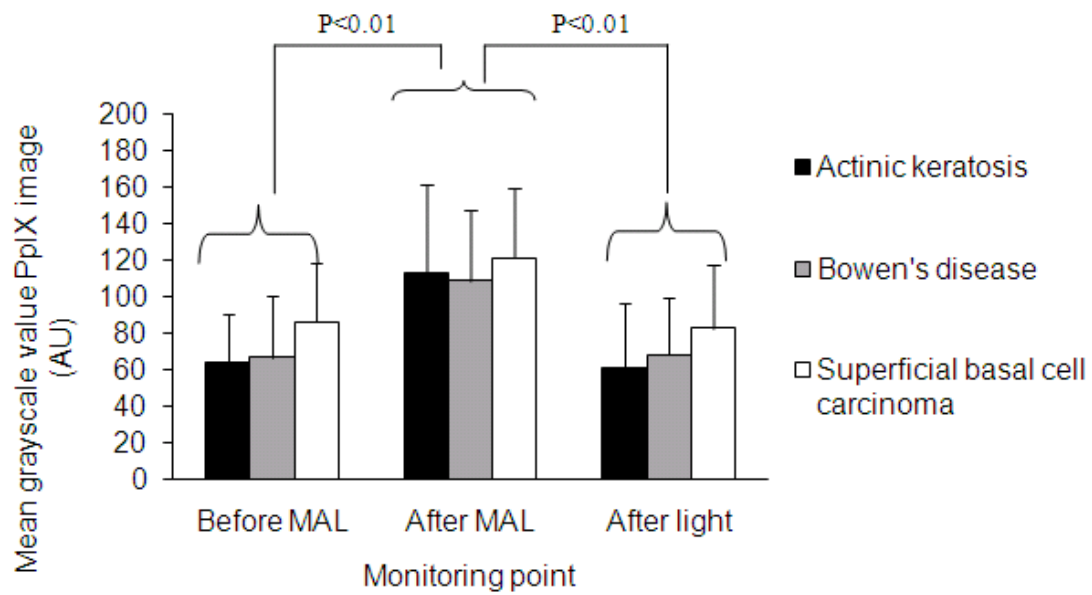


Figure 2

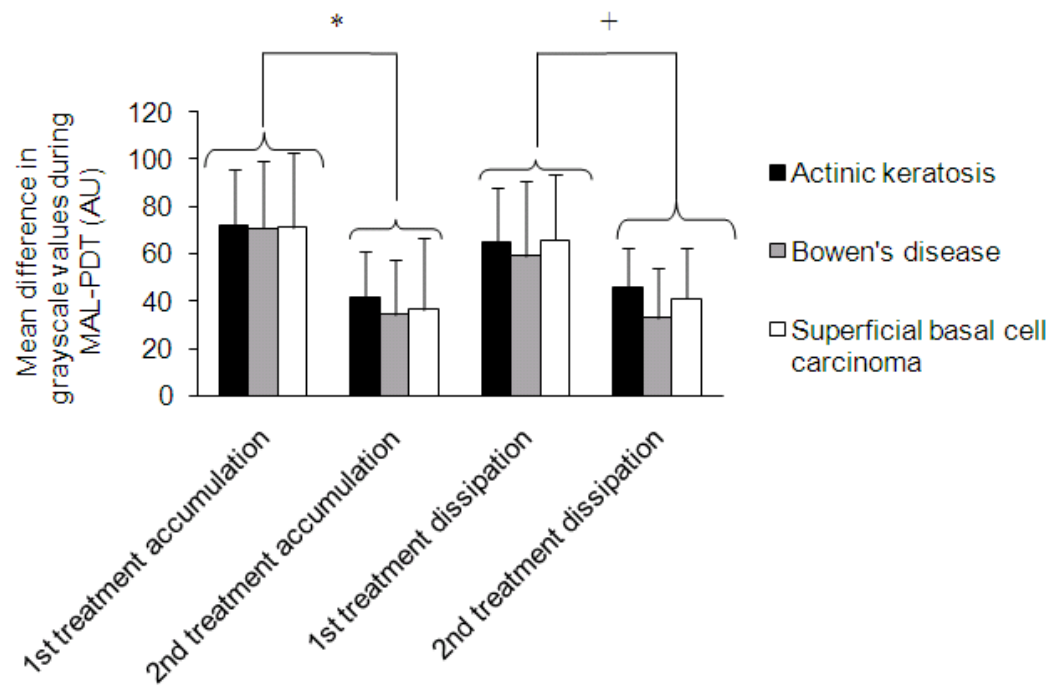


Figure 3

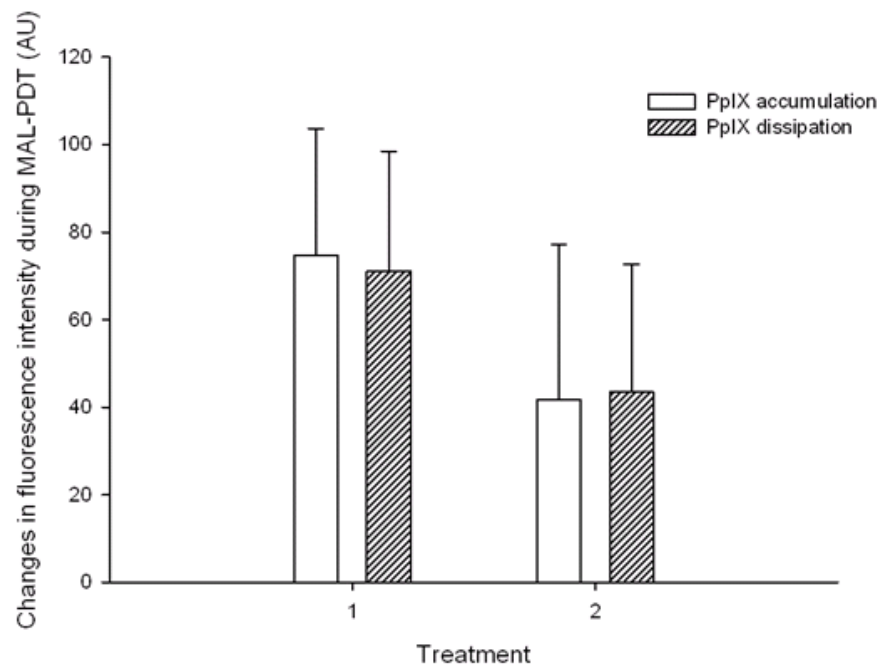


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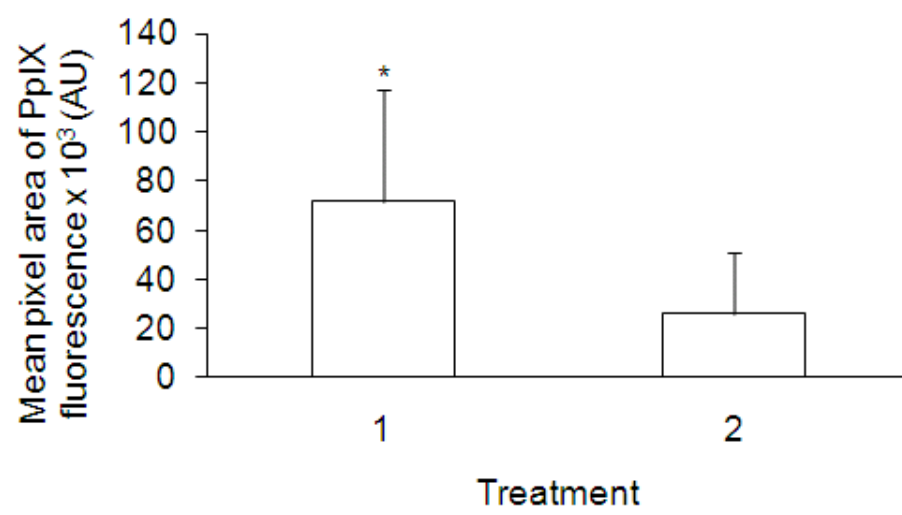


Figure 5

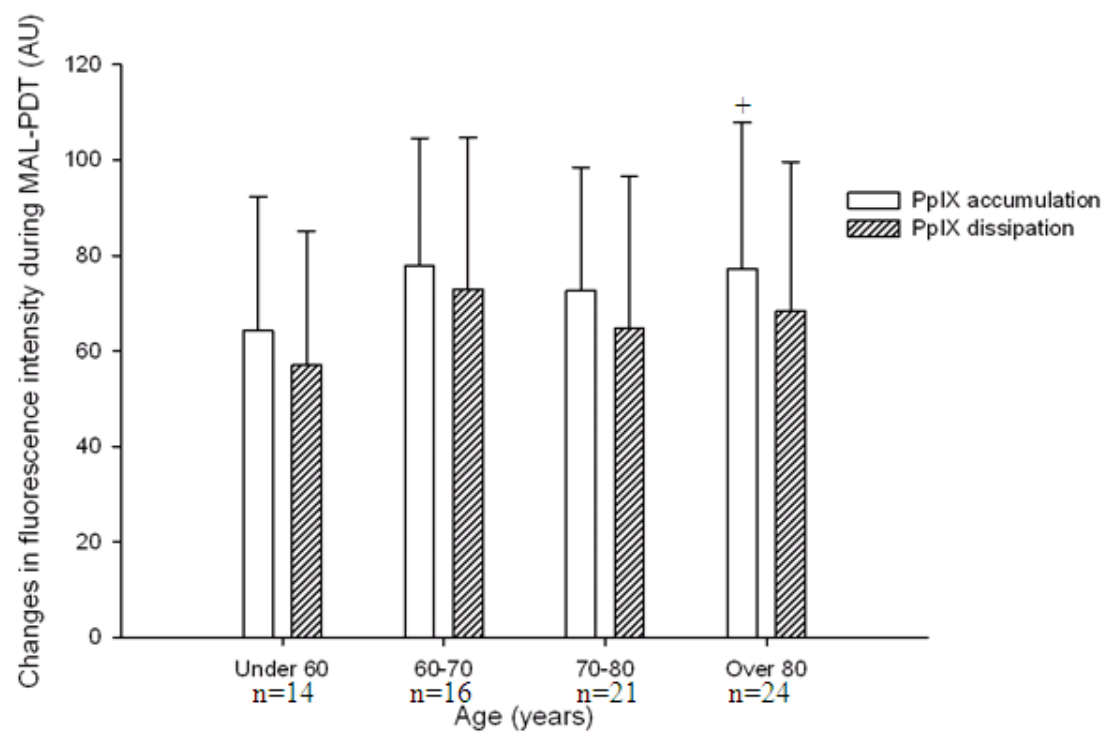


Figure 6

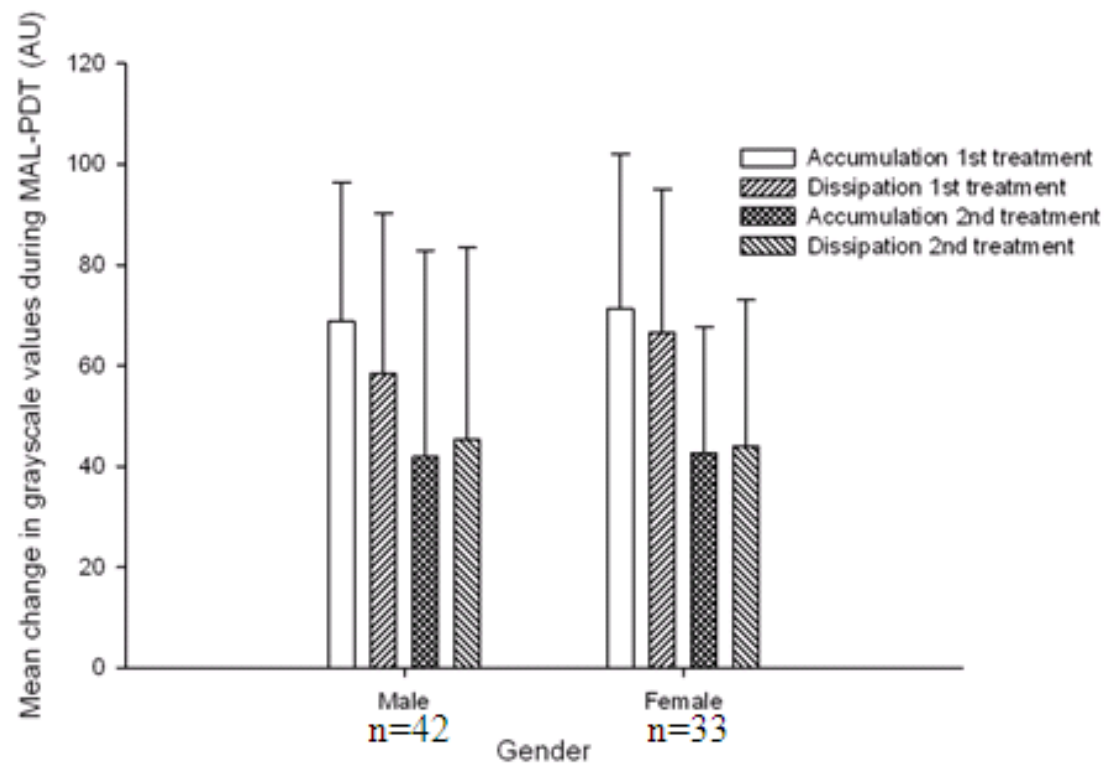


Figure 7



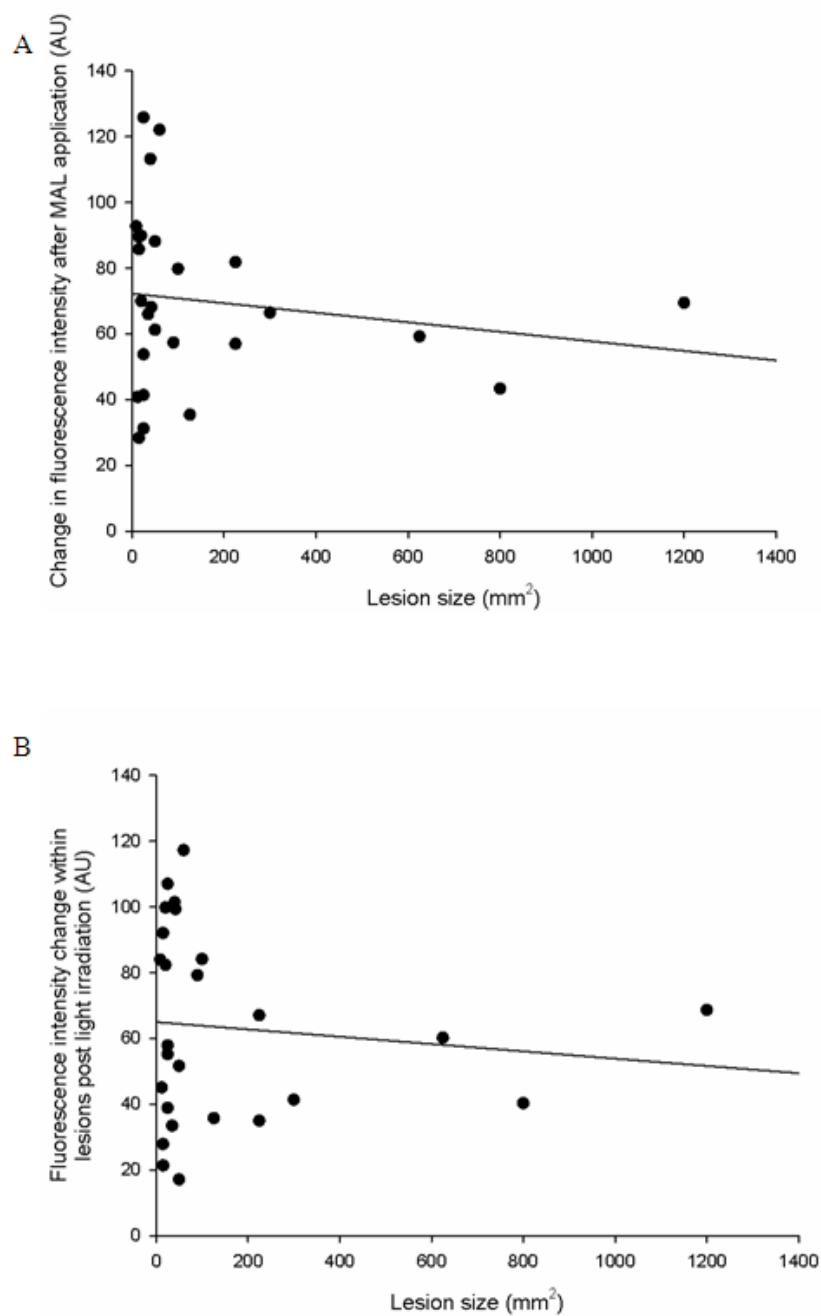


Figure 8